

Schizosaccharomyces pombe Pak-related protein, Pak1p/Orb2p, phosphorylates myosin regulatory light chain to inhibit cytokinesis

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P21-activated kinases (Paks) have been identified in a variety of eukaryotic cells as key effectors of the Cdc42 family of guanosine triphosphatases. Pak kinases play important roles in regulating the filamentous actin cytoskeleton. In this study, we describe a function for the *Schizosaccharomyces pombe* Pak-related protein Pak1p/Orb2p in cytokinesis. Pak1p localizes to the actomyosin ring during mitosis and cytokinesis. Loss of Pak1p function leads to accelerated cytokinesis. Pak1p mediates

phosphorylation of myosin II regulatory light chain Rlc1p at serine residues 35 and 36 in vivo. Interestingly, loss of Pak1p function or substitution of serine 35 and serine 36 of Rlc1p with alanines, thereby mimicking a dephosphorylated state of Rlc1p, leads to defective coordination of mitosis and cytokinesis. This study reveals a new mechanism involving Pak1p kinase that helps ensure the fidelity of cytokinesis.

Introduction

The family of conserved p21-activated kinase (Pak) serine/threonine kinases are key downstream effectors of the Cdc42 family of GTPases (Manser et al., 1994). Pak kinases, like cdc42 GTPase, have been extensively studied and implicated in regulating filamentous actin (F-actin) cytoskeleton and cellular morphogenesis in several organisms. For example, Pak1 kinase activity is essential for cylindrical morphology of *Schizosaccharomyces pombe* (Ottillie et al., 1995), bud morphology in *Saccharomyces cerevisiae* (Weiss et al., 2000), and hyphal morphology in *Candida albicans* (Leberer et al., 1997). Pak kinases are also important for F-actin organization and turnover in mammalian cells (Manser et al., 1997) as well as axonal guidance (Hing et al., 1999) and epithelial polarity (Conder et al., 2007) in *Drosophila melanogaster*. Cytokinesis in *S. cerevisiae* requires Pak kinase for septin collar formation (Cvrckova et al., 1995; Kadota et al., 2004; Versele and Thorner, 2004). Pak kinase has also been implicated in cytokinesis in *Dictyostelium discoideum*, although the mechanism of regulation of cytokinesis by Pak kinase has not been clarified (de la Roche et al., 2005).

In recent years, the fission yeast *S. pombe* has become an attractive model organism for the study of the F-actin cytoskel-

eton, cellular morphogenesis, and cytokinesis. Two Pak kinase-related proteins have been identified in *S. pombe*, of which Pak1p/Orb2p/Shk1p is essential for cell viability (Ottillie et al., 1995) and localizes to the cell ends and to the cell division site (Qyang et al., 2002), whereas the second protein, Pak2p/Shk2p, has been shown to be dispensable for growth and colony formation (Sells et al., 1998; Yang et al., 1998).

In this study, we uncover a novel role for Pak1p kinase in the regulation of cytokinesis in fission yeast. We provide evidence that, in addition to localizing to the cell ends, Pak1p localizes to the actomyosin ring at the cell division site. Pak1p appears to phosphorylate the regulatory light chain of myosin II (Rlc1p) and thereby inhibits cytokinesis until complete segregation of the genetic material.

Results and discussion

Pak1p localizes to the cell division site in early mitosis

To better understand the role of fission yeast Pak1p, we first characterized the intracellular distribution of this protein. A strain in which the *pak1* coding sequences were fused in frame with

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Abbreviations used in this paper: F-actin, filamentous actin; MBP, myelin basic protein; MRLC, myosin regulatory light chain; Pak, p21-activated kinase; SPB, spindle pole body; YES, yeast extract plus supplements.

The online version of this article contains supplemental material.

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the gene encoding GFP was generated. As described previously in a fission yeast strain overexpressing *pak1* (Qyang et al., 2002), wild-type Pak1p was detected at the cell ends of interphase cells (Fig. 1 A, i). In cells undergoing mitosis and cytokinesis, Pak1p was detected at the cell division site. To precisely determine the temporal regulation of Pak1p localization to the cell division site, we characterized its localization in cells expressing Sid4p-GFP, a marker of the spindle pole body (SPB) that also served to monitor cell cycle progression (Chang and Gould, 2000). In cells with two closely spaced SPBs, which is indicative of prometaphase, Pak1p signal was still detected at the cell ends (Fig. 1 A, ii). Interestingly, after prometaphase and before anaphase A, Pak1p relocated from the cell ends to the cell division site (Fig. 1 A, iii). The intensity of the medial signal increased as the cell proceeded through anaphase B (Fig. 1 A, iv). In septating cells, Pak1p signal was present at the septum region (Fig. 1 A, v).

To investigate whether Pak1p localization to the cell division site coincides with the presence of the actomyosin ring in mitosis, a strain coexpressing Pak1p-GFP and Rlc1p-Cherry was imaged (Fig. 1 B). In fission yeast, the actomyosin ring is assembled before anaphase A, whereas its constriction occurs after completion of anaphase B (Wu et al., 2003). This medial Pak1p-GFP signal colocalized with Rlc1p-Cherry (Fig. 1 B, i). During actomyosin ring constriction, Pak1p-GFP was detected in the vicinity of the constricting actomyosin ring as well as with the developing septum (Fig. 1 B, ii). In some tilted view, Pak1p-GFP was found to clearly organize into a ring structure (Fig. 1 B, iii).

To further determine whether Pak1p is associated with the actomyosin ring, we examined its localization in β -1,3 glucan synthase *cps1*-191 mutant that is defective in septum assembly at the nonpermissive temperature (Liu et al., 1999). At 36°C, most cells arrested with an actomyosin ring (Rlc1p-Cherry signal) and Pak1p-GFP were observed to localize at the division site (Fig. 1 C). Similarly, in β -tubulin *nda3*-KM311 mutant at the nonpermissive temperature, Pak1p-GFP was localized at the division site in these prometaphase-arrested cells (42% of the cell population arrested in prometaphase, $n = 200$; Fig. 1 D). These experiments established that Pak1p was localized in the vicinity of the actomyosin ring as well as the division septum, suggesting a potential role in the regulation of cytokinesis.

A hypomorphic *pak1* allele promotes early actomyosin ring constriction

The available mutant alleles of *pak1* were substantially compromised for cell shape and were unsuitable for the study of cytokinesis. Therefore, we created a mutant allele of *pak1* (*pak1*-M460G) in which the ATP-binding site was mutated. The kinase activity of Pak1p-M460G relative to that of wild-type Pak1p was measured with myelin basic protein (MBP) phosphorylation (Fig. 1 E). This analysis established that the kinase activity of Pak1p-M460G was significantly reduced as compared with that of Pak1p.

The *pak1*-M460G strain was viable at 18–36°C laboratory conditions (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200806127/DC1>) but displayed defects in growth pat-

terning (Fig. 1 F). Pak1p-M460G-expressing cells exhibited a monopolar growth pattern. After cell separation, one daughter cell grew from the new end (Fig. 1 F, white arrows), whereas the other daughter cell grew from the old end (Fig. 1 F, black arrows). Interestingly, Pak1p-M460G was capable of localizing to the growing zones and to the cell division site (Fig. 1 F, 180'), suggesting that maximal kinase activity of Pak1p is not essential for its cellular localizations. Pak1p-M460G was present at the division site in 37% of cell populations ($n = 200$) that represented the prometaphase-arrested cells under these conditions (Fig. 1 G).

To understand the role of Pak1p function in cytokinesis, we studied the dynamics of actomyosin ring assembly and constriction in wild-type and *pak1*-M460G cells expressing Rlc1p-GFP (actomyosin ring marker) and Sid4p-GFP (SPB marker) by time-lapse microscopy. Kymographs of the Rlc1p-GFP actomyosin ring beginning at early anaphase (defined by two SPBs at 5–7 μ m apart) are shown in Fig. 2 A. Assembly of the actomyosin ring was not perturbed in *pak1*-M460G cells in that early anaphase cells contained normal actomyosin rings. Interestingly, whereas the process of cytokinesis lasted a mean value of 36.2 min in wild-type cells, the process was considerably faster with a mean value of 31.6 min in *pak1*-M460G-expressing cells (Fig. 2, A and B). Mitotic spindle elongation rates in wild-type- and *pak1*-M460G-expressing cells were comparable (unpublished data), suggesting that the accelerated cytokinesis in *pak1*-M460G cells was not caused by accelerated mitotic progression.

We considered two possible explanations for the observed accelerated cytokinesis in *pak1*-M460G-expressing cells. First, it was possible that actomyosin ring constriction was accelerated in *pak1*-M460G cells. Second, it was possible that actomyosin ring constriction was initiated earlier than normal in *pak1*-M460G cells. Kymographic analyses revealed that the mean time of ring constriction was similar in wild-type and *pak1*-M460G cells, but the initiation of ring constriction seemed to be earlier in *pak1*-M460G cells (Fig. 2 A, arrows).

To confirm our analysis that the actomyosin ring constriction was initiated earlier in *pak1*-M460G cells, we synchronized cells in prometaphase using the cold-sensitive *nda3*-KM311 mutant. Under these conditions, *nda3*-KM311 and the *nda3*-KM311 *pak1*-M460G mutants arrested in prometaphase with stable actomyosin rings. Return of the arrested cells to the permissive temperature led to spindle assembly and a synchronous resumption of downstream events of mitosis, such as chromosome segregation and cytokinesis (Fig. 2 C). In *nda3*-KM311 cells (Fig. 2 D), as in wild-type cells, actomyosin ring constriction was largely initiated upon dissolution of the mitotic spindle. In contrast, actomyosin ring constriction in the majority of *nda3*-KM311 *pak1*-M460G cells was initiated when the mitotic spindle was still present (Fig. 2, D and E). Collectively, these experiments established that compromise of Pak1p kinase function led to premature constriction of the actomyosin ring in anaphase cells.

Coregulation of Pak1p and Rlc1p

To understand the molecular mechanism underlying *pak1* kinase regulation of cytokinesis, we took a candidate approach to identify potential substrates whose phosphorylation might regulate

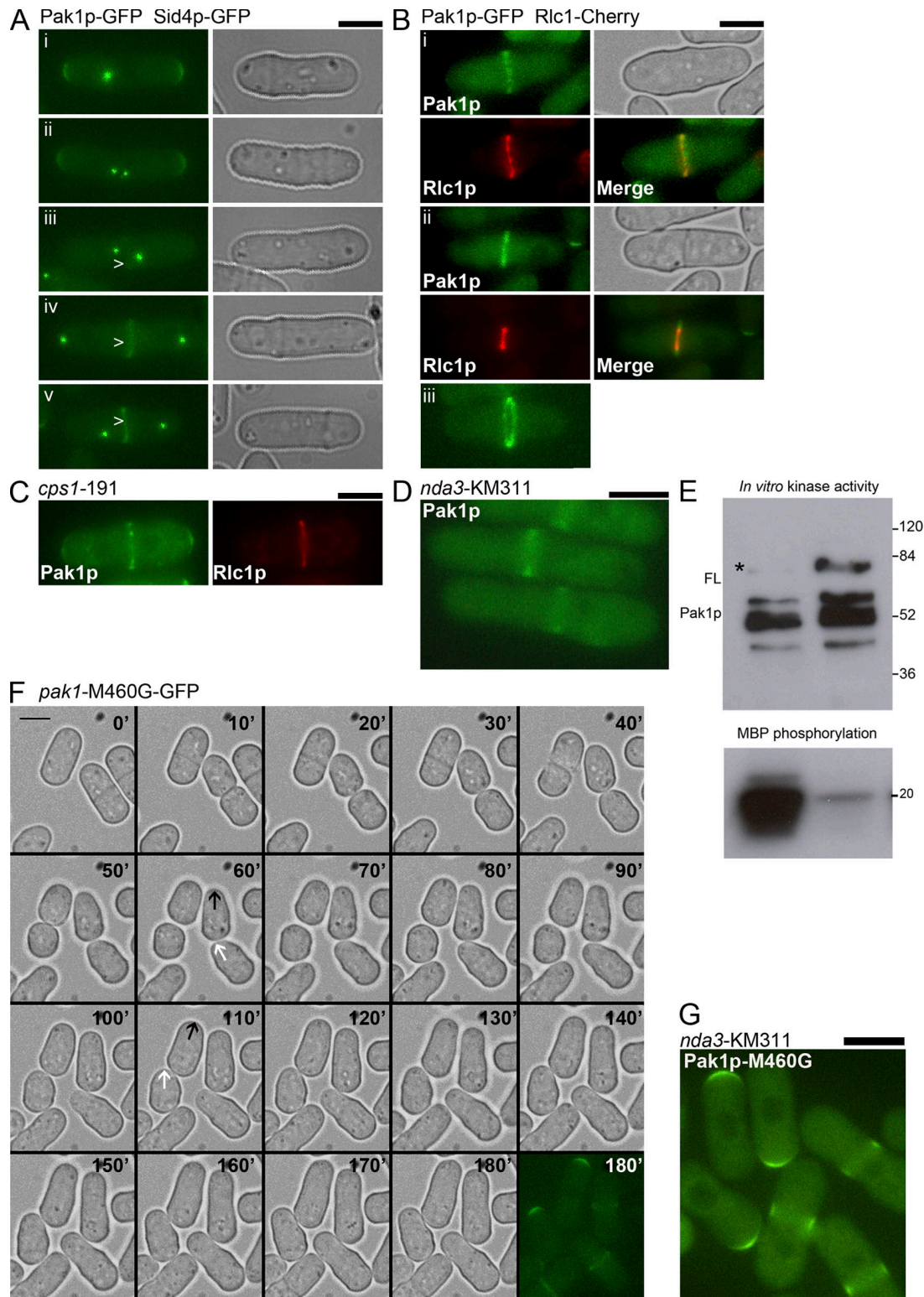
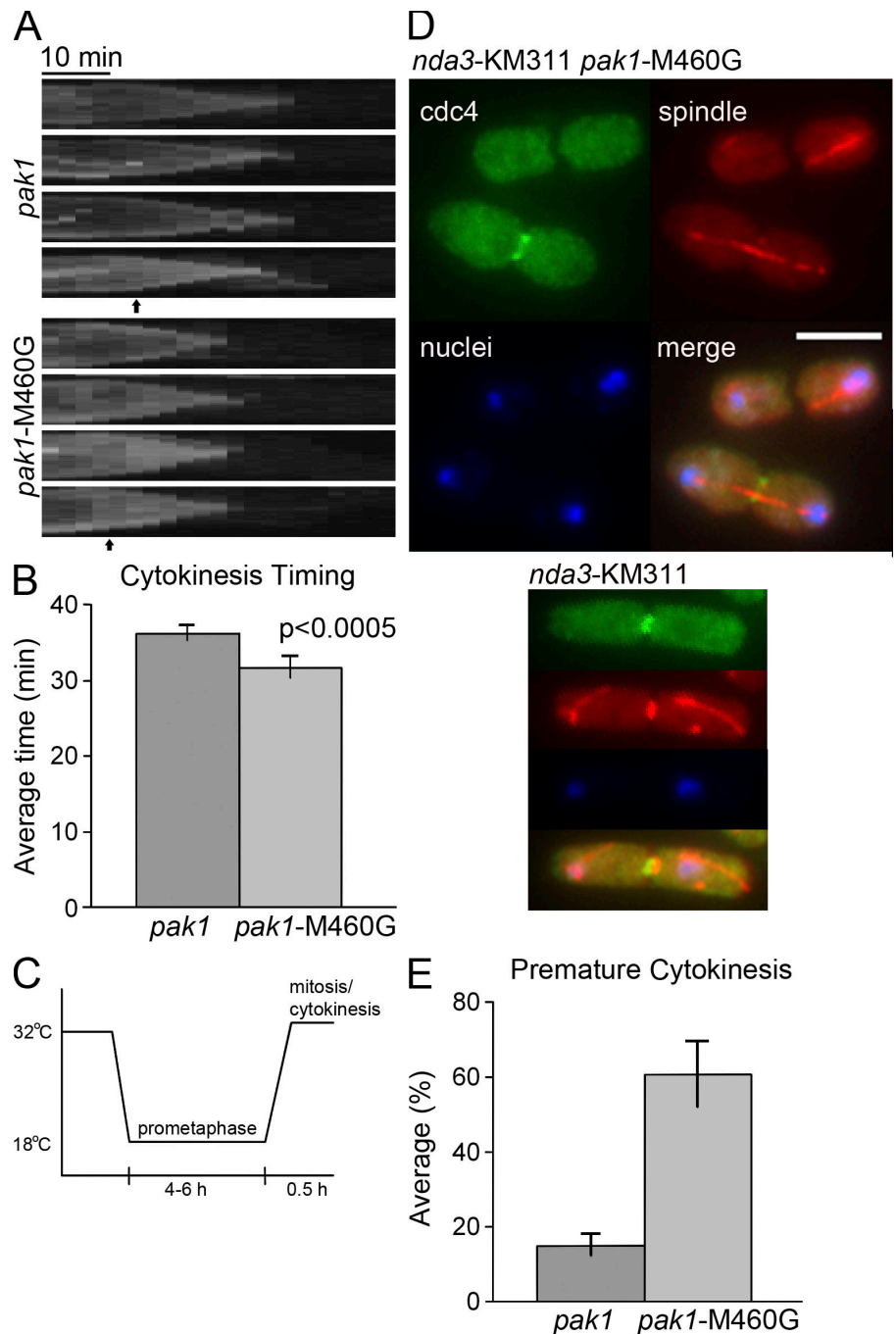


Figure 1. **Pak1p localizes to the growing cell ends in interphase and to actomyosin ring in mitosis.** (A) Cell images of Pak1p-GFP and Sid4p-GFP (a marker for the SPB and for cell cycle progression). Pak1p-GFP signals at the division site are marked with arrowheads. (B) Cell images of Pak1p-GFP and Rlc1p-Cherry (a marker for the actomyosin ring). (C) Cell images of *cps1-191* strain expressing Pak1p-GFP and Rlc1p-Cherry. The *cps1-191* strain was incubated at 36°C for 4 h before imaging. (D) Cell image of *nda3-KM311* strain expressing Pak1p-GFP. The *nda3-KM311* strain was incubated at 18°C for 6 h before imaging. (E) *In vitro* kinase assay of Pak1p and Pak1p-M460G. Pak1p was immunoprecipitated with anti-Myc antibodies from fission yeast lysate and incubated with exogenous MBP substrate for kinase activity assessment. Pak1p existed as full length (FL; *, Pak1p-GFP 98 kD) and several smaller protein bands in the Western blot analysis. (F) Time-lapse images of *pak1-M460G-GFP* strain. Pak1p-GFP signal was captured in the last image. After cell separation, one daughter cell grew from the new end (white arrows), whereas the other daughter cell grew from the old end (black arrows). (G) Cell image of *nda3-KM311* strain expressing Pak1p-M460G-GFP (culture condition as in D). Bars, 5 μ m.

Figure 2. **Cytokinesis in *pak1*-M460G proceeds with faster kinetics.** (A) Kymographs of the actomyosin ring of wild-type and *pak1*-M460G strains. The onset of ring constriction is marked with arrows. (B) The mean time taken for the actomyosin ring from early anaphase to the completion of ring constriction was quantified from time-lapse images ($n = 30$). (C) Schematic diagram of *nda3*-KM311 strains expressing either Pak1p-GFP or Pak1p-M460G-GFP at 18°C for 4–6 h to synchronize cells in prometaphase. Upon shifting to 32°C for 30 min, cultures were fixed for immunostaining. (D) Images of *cdc4* (a marker for the actomyosin ring), mitotic spindle, and DAPI (nucleus). Bar, 5 μ m. (E) Cells with a constricting *cdc4* actomyosin ring were counted; a higher percentage of Pak1p-M460G-expressing cells was in anaphase with a mitotic spindle. The mean value was derived from three experiments, and 300 cells were counted in each experiment. Error bars indicate SD.



actomyosin ring dynamics. Mammalian pak1 inhibits myosin II activity by inhibiting myosin light chain kinase and thereby reducing the activating phosphorylation of its myosin regulatory light chain (MRLC; Sanders et al., 1999). Conversely, a previous study has also shown that mammalian Pak can directly phosphorylate MRLC and activates myosin II (Chew et al., 1998). The amino acids in *S. pombe* Rlc1p that are equivalent to the phosphorylation sites in mammalian MRLC are shown in Fig. S2 (available at <http://www.jcb.org/cgi/content/full/jcb.200806127/DC1>). The codons for the serine residues 35 and 36 in *S. pombe rlc1* were substituted with those for alanine (*rlc1*-S35AS356A [SA]). *S. pombe* Pak1p kinase, Rlc1p, and Rlc1p-SA were expressed in bacteria and purified. In vitro ki-

nase assays revealed that Pak1p kinase phosphorylated wild-type Rlc1p but not Rlc1p-SA (Fig. 3 A). We further analyzed Rlc1p phosphorylation in vivo in synchronized early anaphase cells. By isoelectric focusing and SDS-PAGE analyses, Rlc1p from wild-type cells was resolved into two spots (Fig. 3 B, arrowheads) with the smaller spot (nearer to pH 3), possibly a phosphorylated form (Fig. 3 B, top). Interestingly, Rlc1p from *pak1*-M460G cells was resolved as one spot indicating lack of protein modification (Fig. 3 B, bottom). Thus, Rlc1p is directly phosphorylated either by Pak1 kinase or by another kinase downstream of Pak1 in vivo.

Protein–protein interaction of Pak and its substrate has been shown to be important for its localization in mammalian

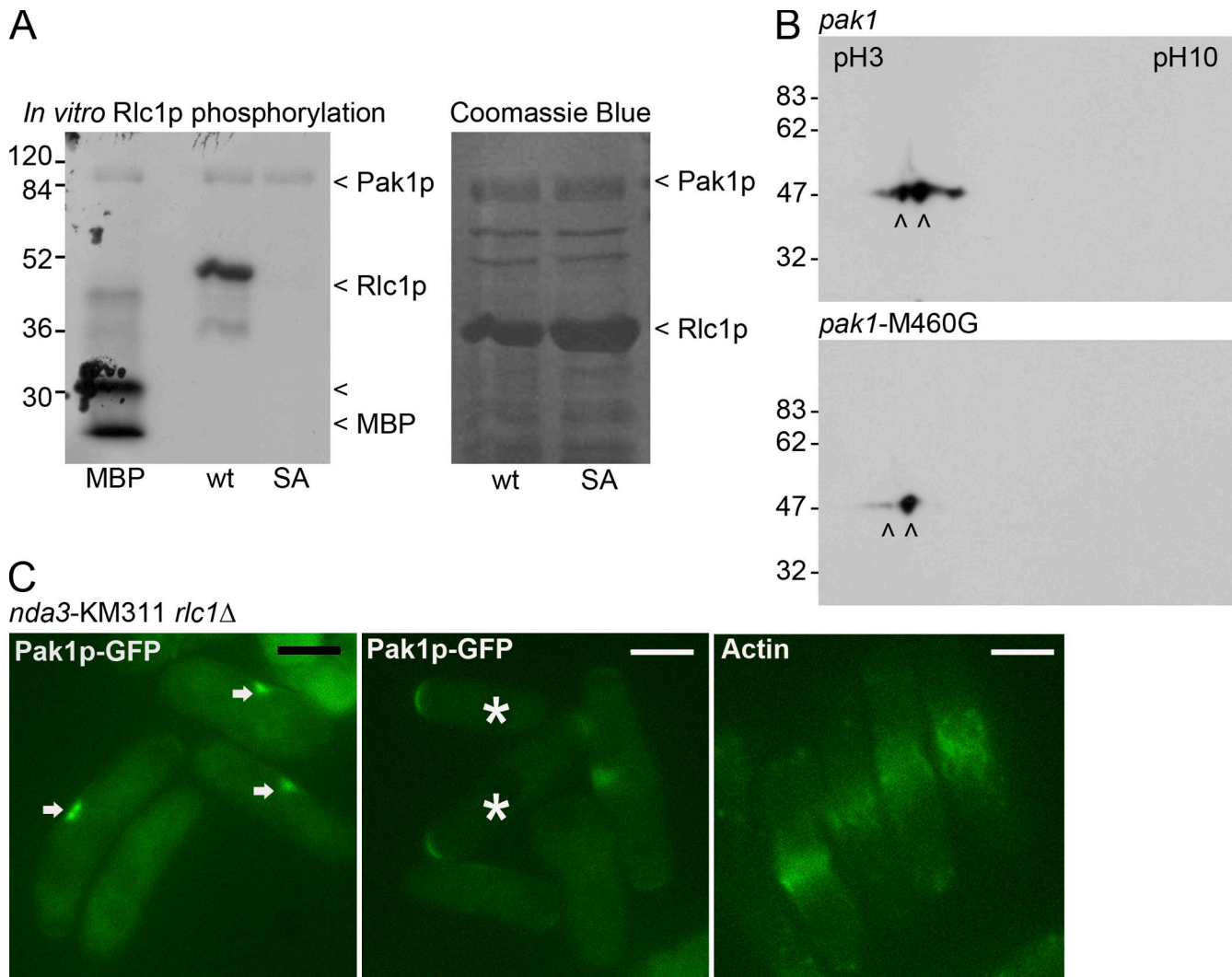


Figure 3. Pak1p phosphorylates Rlc1p in vitro and in vivo, and Pak1p localization to the actomyosin ring is dependent on Rlc1p. (A) Recombinant Pak1p (GST-Pak1p 98 kD) was incubated with MBP, Rlc1p (GST-Rlc1p 47 kD), or Rlc1p-S35AS36A in the presence of γ -[32 P]ATP. The left panel shows Pak1p autophosphorylation and Pak1p phosphorylation of wild-type (wt) Rlc1p but not the Rlc1p-SA mutant. The right panel shows Pak1p and Rlc1p proteins in this assay. (B) 2D SDS-PAGE resolution of Rlc1p-Myc (arrowheads) in early anaphase cells of wild-type (top) and *pak1-M460G* strains (bottom). (C) Cell images of Pak1p-GFP in prometaphase cells (arrows) and in interphase cells (asterisks) of the *nda3-KM311 rlc1Δ*-null strain at a nonpermissive temperature. These cells were also fixed and stained with phalloidin Alexa Fluor 488. Bars, 5 μ m.

cells (Manser et al., 1998). Because Rlc1p is a putative substrate of Pak1p, we tested whether Pak1p-GFP localization to the actomyosin ring depended on Rlc1p function. To this end, *nda3-KM311 rlc1Δ* cells were arrested at prometaphase, and Pak1p-GFP localization was monitored. In these *rlc1Δ* cells, the actomyosin ring was less compacted (Fig. 3 C, actin). In *nda3-KM311* and *nda3-KM311 rlc1Δ* cells, F-actin was observed in medial rings and bands in 65–75% of cells. However, Pak1p-GFP was observed in these medial rings and band structures in <5% of cells and was largely detected as aggregates in 63% of the *nda3-KM311 rlc1Δ* cells ($n = 200$; Fig. 3 C, arrows). The fact that Pak1p localization to cell ends (Fig. 3 C, asterisks) was not affected established that loss of Rlc1p function was not generally destabilizing the localization of Pak1p at all cellular locations but was specific for its effects on the actomyosin ring. Collectively, these experiments established that Pak1p likely phosphorylates Rlc1p at serine 35 and 36 and that the putative substrate

played an important role in the localization of the kinase to the actomyosin ring.

Pak1 kinase and Rlc1p phosphorylation inhibit actomyosin ring constriction until completion of mitosis

We have shown that cells compromised for Pak1p function undergo premature constriction of the actomyosin ring and that Pak1p phosphorylates Rlc1p on serine 35 and 36 both in vitro and in vivo. We investigated the physiological consequences resulting from compromised Pak1p function. In fission yeast cells, the actomyosin ring is assembled upon entry into mitosis and constricts upon completion of anaphase B and spindle dissolution. Prometaphase-arrested *nda3-KM311 pak1-M460G* cells did not undergo actomyosin ring constriction and septation (unpublished data). Thus, Pak1p function was not required to restrain cytokinesis in metaphase-blocked cells.

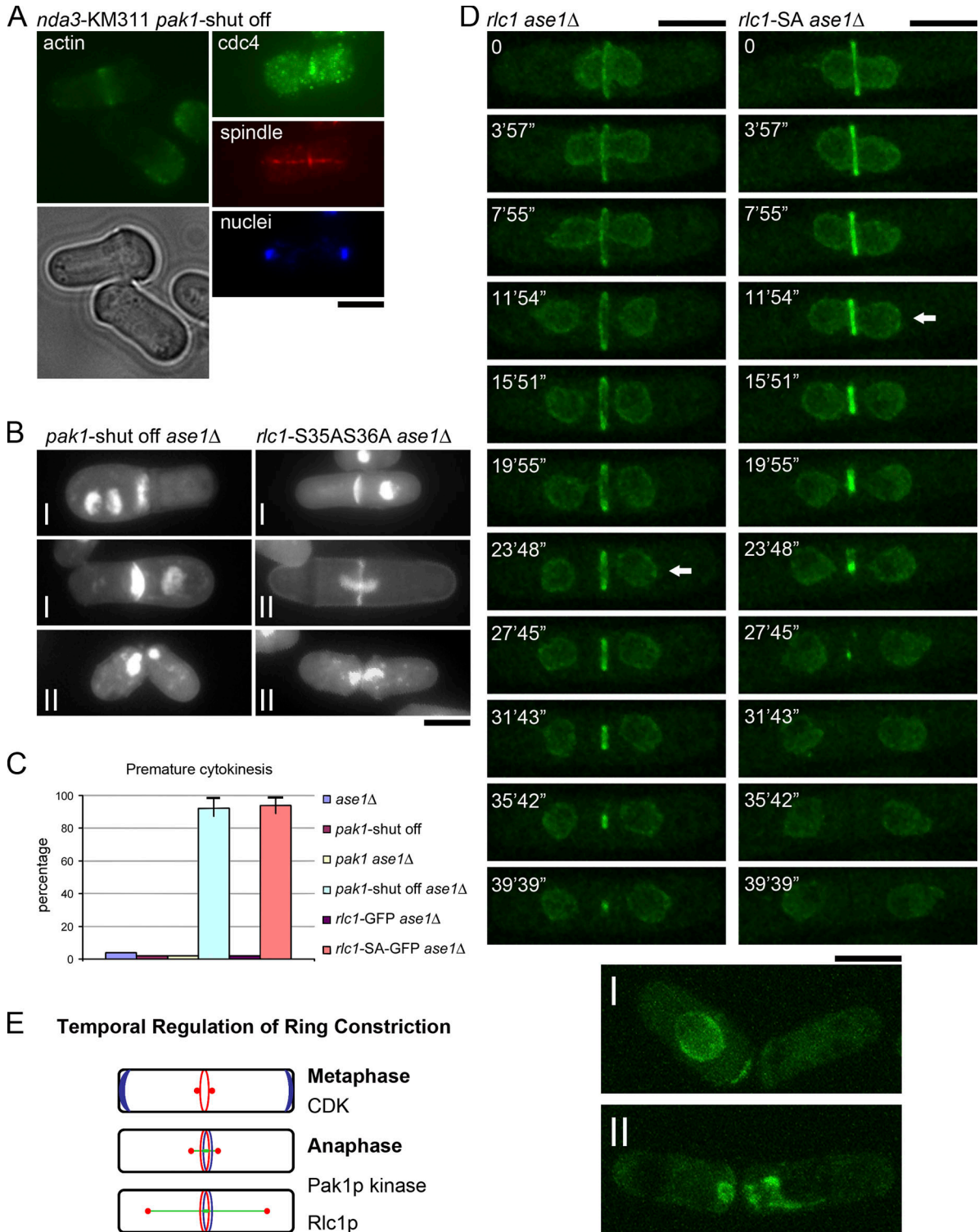


Figure 4. **Pak1p kinase and Rlc1p phosphorylation are essential for inhibiting cytokinesis when mitotic progression is slowed down.** (A) The *nda3-KM311 nmt1-pak1* strain was grown in YES (+15 μ M thiamine to shut off *pak1* expression). After 6-h incubation at 18°C, some cells were fixed and stained with phalloidin Alexa Fluor 488. Actin patches were present at one cell end in interphase cells, indicative of monopolar growth. Other cells were arrested in prometaphase with an actomyosin ring (left). The remaining culture was returned to the permissive temperature at 32°C for 30 min, and cells were fixed and stained with anti-*cdc4* (actomyosin ring), anti-TAT1 (spindle), and DAPI (nucleus; right). Premature ring constriction was evident in the *pak1* shut off strain. (B) *ase1*-null and *nmt1-pak1 ase1*-null strains were inoculated into YES (+15 μ M thiamine to shut off *pak1* expression) for 9 h. *rlc1-GFP ase1* null and *rlc1-SA-GFP ase1* null were cultured in YES medium at 24°C. These cultures were fixed and stained with DAPI (nucleus) and aniline blue (septum) for scoring of septation phenotype. (C) Quantification of septation phenotype. The percentage value was the mean of three experiments, and 300 cells were counted in each experiment. Error bars indicate SD. (D) Time-lapse images of *ase1* null-expressing Rlc1p-GFP and Rlc1p-SA-GFP (with Uch2p-GFP as a nuclear marker) during mitotic progression and cytokinesis. Arrows indicate initiation of ring constriction. (E) Model shows that *pak1* kinase and *rlc1* phosphorylation coordinate mitotic progression and cytokinesis initiation. Pak1p (blue ovals), actomyosin ring (red ovals), SPBs (red dots), and spindle (green lines) in anaphase cells. Bars, 5 μ m.

We tested whether cells that undergo slower anaphase progression would depend on Pak1p function to restrain actomyosin ring constriction and septation. To this end, we created double mutants defective in Ase1p, a protein important for spindle integrity and nuclear segregation (Liodice et al., 2005), and *pak1*-M460G. This strain exhibited a deleterious genetic interaction and was not viable (unpublished data). As a result, *nmt1-pak1* (*pak1* expression regulated by *nmt1* promoter at its genomic locus) strain was used in place of *pak1*-M460G for this analysis. *nmt1-pak1* cells were similar to *pak1*-M460G cells (Fig. 4 A). We tested genetic interactions and cytokinesis timing in *nmt1-pak1 ase1Δ* double mutants. Interestingly, *nmt1-pak1 ase1Δ* double mutants displayed defective cytokinesis characterized by the presence of fully formed septa before full segregation of the chromosomes. A similar effect was observed in double mutants defective in *ase1* and *rlc1*-SA (in which serine 35 and 36 were replaced with alanine; Fig. 4 B). Quantification of these experiments is shown in Fig. 4 C. Live cell imaging of *ase1Δ* and *rlc1*-SA *ase1Δ* double mutants was also performed to determine the temporal regulation of ring constriction (Fig. 4 D). In *ase1Δ* cells, actomyosin ring constriction (followed using Rlc1p-GFP as a marker) was initiated at least 20 min after ring assembly and nuclear segregation. However, in the double mutant, ring constriction was initiated earlier and before nuclear segregation, leading to a variety of aneuploidy events, including the formation of anucleate cells in several instances. The phenotype is classified into two major types: (a) septated cell with the nucleus displaced to one compartment and (b) septation through the segregating nuclei. Collectively, these experiments suggested that Pak1p by phosphorylating Rlc1p might function to delay actomyosin ring constriction and septum assembly until completion of chromosome segregation and spindle disassembly.

In summary, we have shown that fission yeast Pak1p localizes to the actomyosin ring during early mitosis. Pak1p localization is similar to that of Rga8p, a Rho-GTPase-activating protein that interacts with Pak1p (Yang et al., 2003). It is presently unclear whether the Rho-GTPase-activating protein Rga8p might participate in the regulation of cytokinesis as well. Although compromising Pak1p function did not affect the timely assembly of actomyosin rings, actomyosin ring constriction was prematurely initiated in the *pak1*-M460G mutant. We have shown that Pak1p phosphorylates serine 35 and 36 of Rlc1p, the regulatory light chain of fission yeast myosin II. Compromising Pak1p function or Rlc1p function (by replacement of serine 35 and 36 with the nonphosphorylatable amino acid alanine) in cells that are slowed down in anaphase leads to aneuploidy caused by premature ring constriction. However, premature ring constriction was not observed upon compromise of Pak1p or Rlc1p function in prometaphase- and/or metaphase-arrested cells. These observations suggest that at least two mechanisms might exist to prevent actomyosin ring constriction until completion of chromosome segregation. The first mechanism might involve prevention of activation of the septation initiation network in metaphase by high levels of Cdk activity (Guertin et al., 2000). Our current experiments reveal that Pak1p, via Rlc1p phosphorylation, might prevent actomyosin ring constriction in anaphase A and B cells, thereby contributing to genomic stability (Fig. 4 E).

In metazoans, phosphorylation of the major isoform MRLC at amino acids (equivalent to serine 35 and 36 of the fission yeast Rlc1p) leads to activation of myosin II function and appears to regulate cytokinesis (Yamakita et al., 1994; Matsumura et al., 1998). In contrast, our experiments suggest the possibility that phosphorylation of Rlc1p at serine 35 and 36 leads to down-regulation of myosin II function. Although these observations appear to contradict each other, it is possible that other MRLC isoforms might exist in animal cells whose phosphorylation might prevent myosin II activation. Future studies should test this possibility.

Materials and methods

pak1 plasmids and strains construction

Pak1p-GFP has its 5' start codon replaced with a stop codon, and its 3' stop codon is replaced with a six-glycine linker and two copies of Myc epitopes and is cloned in frame to GFP. Mutations were generated by site-directed mutagenesis (Agilent Technologies). Plasmids were linearized with XhoI and integrated into the *pak1* locus by homologous recombination; its expression was under the control of the native promoter. The *nmt1-pak1* strain was constructed by PCR-based targeting at *pak1* genomic locus (Bahler et al., 1998).

Genetic and cell biological techniques

S. pombe strains used in this study are listed in Table I. The *ase1*-null strain was provided by S. Olfiferenko (Temasek LifeSciences Laboratory, Singapore). All Pak1p-GFP-expressing strains were grown in rich yeast extract plus supplements (YES) media, wild-type and Pak1p-M460G-expressing cells were grown at 24°C to mid-log phase, and strains in *cps1*-191 and *nda3*-KM311 genetic background were grown at temperatures specified in the figure legends. Cells were spotted on YES 2% agarose pad, and imaging was performed with a microscope with a Plan Apo 100×/1.45 NA oil lens (IX71; Olympus) equipped with a camera (CoolSnap ES; Photometrics) and MetaMorph software (version 6.2r6; MDS Analytical Technologies) at 22°C. *cdc25-22* cells were grown at 24°C to mid-log phase, were shifted to 36°C for 4 h to arrest cells in G2, and were shifted down to 24°C to allow synchronous entry into mitosis. Conditions for time-lapse imaging were performed according to the protocol described previously (Tran et al., 2004) with a confocal microscope (LSM510; Carl Zeiss, Inc.) with a 24°C heated Plan-Neofluor 100×/1.30 NA oil lens. Images were converted into a 3D stack with LSM Image Browser (version 3.5.0.376; Carl Zeiss, Inc.), and a kymograph was generated with MetaMorph (version 6.1). *nda3*-KM311 strains were methanol fixed for indirect immunofluorescence staining with mouse anti-TAT1 (provided by K. Gull, University of Manchester, Manchester, England, UK) and rabbit anti-*cdc4* antibodies at 1:200 dilutions and secondary antibodies Alexa Fluor 488 and 594 conjugate. Images were captured with a microscope (DM IRE2; Leica) with a 100×/1.4 NA oil lens equipped with a CoolSnap ES camera and MetaVue software (version 6.2r4; MDS Analytical Technologies). *nmt1-pak1* and *nmt1-pak1 ase1*-null strains were grown in minimum medium liquid media at 24°C, and subsequently these cultures were split into two halves: one half remaining in minimum medium liquid and the other half grown in YES (supplemented with 15 μM thiamine) to repress *pak1* expression. *rlc1 ase1*-null strains were grown in YES liquid media at 24°C.

In vitro Pak1p kinase activity and Rlc1p phosphorylation

Recombinant GST-Pak1p, GST-Rlc1p, and GST-Rlc1p-S35AS36A were expressed in BL21 DE3 bacteria strain and purified. 1 μg Pak1p kinase was incubated in kinase buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 10 mM MgCl₂, and 1 mM MnCl₂) containing 10 μCi of freshly added γ-³²PATP, 10 μM cold ATP, and substrate (20 μg Rlc1p, 20 μg Rlc1p-S35AS36A, or 2.5 μg MBP) at 22°C for 30 min. The reaction was terminated with SDS sample buffer and resolved with SDS-PAGE for autoradiography.

In vivo Rlc1p-Myc phosphorylation analysis by 2D SDS-PAGE

cdc25-22 cells were harvested 30 min after synchronous entry into mitosis. Cells were lysed by glass bead disruption in buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 20 mM β-glycerophosphate, 1% Triton X-100, 100 mM okadaic acid, 1 mM DTT, protease inhibitor cocktail, and PMSF). An immobilized pH gradient strip (Bio-Rad Laboratories), pH 3–10, was rehydrated with 160 μg of clarified protein lysate overnight before focusing. The immobilized pH gradient strip was transferred

Table I. List of fission yeast strains constructed in this study

Name	Genotype
MBY4489	<i>h- pak1-2xMyc-GFP::ura4*</i>
MBY5064	<i>h- pak1-M460G-2xMyc-GFP::ura4*</i>
MBY4694	<i>h+ pak1-2xMyc-GFP::ura4* sid4-GFP::kan^R</i>
MBY5163	<i>h- pak1-2xMyc-GFP::ura4* rlc1-Cherry::ura4*</i>
MBY5187	<i>h- cps1-191 pak1-2xMyc-GFP::ura4* rlc1-Cherry::ura4*</i>
MBY4542	<i>h- nda3-KM311 pak1-2xMyc-GFP::ura4*</i>
MBY5252	<i>h- nda3-KM311 pak1-M460G-2xMyc-GFP::ura4*</i>
MBY5177	<i>h+ cdc25-22 rlc1-GFP::ura4* sid4-GFP::kanR</i>
MBY5178	<i>h+ cdc25-22 pak1-M460G::ura4* rlc1-GFP::ura4* sid4-GFP::kanR</i>
MBY5290	<i>h+ nda3-KM311 pak1-2xMyc-GFP::ura4* rlc1-GFP::ura4*</i>
MBY5291	<i>h+ nda3-KM311 pak1-M460G-2xMyc-GFP::ura4* rlc1-GFP::ura4*</i>
MBY5286	<i>h- nda3-KM311 pak1-2xMyc-GFP::ura4* rlc1::ura4*</i>
MBY5175	<i>h- cdc25-22 rlc1-MYC::ura4* sid4-GFP::kanR</i>
MBY5176	<i>h- cdc25-22 pak1-M460G::ura4* rlc1-Myc::ura4* sid4-GFP::kanR</i>
MBY5706	<i>h+ nda3-KM311 nmt1-3xHA-pak1-13xMyc</i>
MBY5678	<i>h- ase1::ura4* rlc1-GFP::ura4*</i>
MBY5679	<i>h- ase1::ura4* rlc1-S35AS36A-GFP::ura4*</i>
MBY5670	<i>h- nmt1-3xHA-pak1-13xMyc</i>
MBY5671	<i>h- ase1::ura4* nmt1-3xHA-pak1-13xMyc</i>
MBY5693	<i>h+ ase1::ura4* rlc1-S35AS36A-GFP::ura4* uch2-GFP::ura4*</i>
MBY5698	<i>h- ase1::ura4* rlc1-GFP::ura4* uch2-GFP::ura4*</i>
MBY5001	<i>h- pak1-K415R-2xMyc-GFP::ura4*</i>
MBY3451	<i>h- nmt1-3xHA-pak1</i>
MBY3540	<i>h- nmt1-3xHA-pak1-K415R</i>

to 10% SDS-PAGE. Western blotting was performed with anti-Myc antibodies and protein A-HRP conjugate.

Online supplemental material

Fig. S1 shows that kinase-compromised *pak1* alleles were viable. Fig. S2 shows the sequence alignment of mammalian MRLC and *S. pombe* Rlc1p from the eukaryotic orthology (YOGY) database. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200806127/DC1>.

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